AD_____

GRANT NO: DAMD17-94-J-4351

TITLE: Isolation of a Receptor for WNT/Wingless Growth Factors

PRINCIPAL INVESTIGATOR(S): Roel Nusse, Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-4125

REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1995	Annual 1 Sep 94 - 31 Aug 95

4. TITLE AND SUBTITLE
Isolation of a Receptor for WNT/Wingless Growth Factors

5. FUNDING NUMBERS
DAMD17-94-J-4351

6. AUTHOR(S)
Roel Nusse, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305-4125

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The work done in this grant aims at elucidating the function of the *Wnt* signaling molecules in cancer. *Wnt* proteins are secreted and play important growth controlling roles, in particular in the mammary gland. They can act as oncogenes in mouse mammary tumors. The work is specifically aimed at identifying a receptor for *Wnt* proteins. The isolation of a receptor is critical to our understanding of normal growth control of the mammary gland. Using genetic and biochemical approaches we wish to identify and to clone the receptor for a *Wnt* gene product in Drosophila, called wingless. The receptor will be characterized and using the receptor gene, we will subsequently clone mammalian *Wnt* receptors by homology.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer Oncogenes, molecular biology of breast cancer, growth factors, receptors, signalling between cells, Drosophila, genetics	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet *optical scanning requirements*.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	PR	- Project
G	- Grant	TA	- Task
PE	- Program Element	WU	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

- **DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."
- **DOE** - See authorities.
- **NASA** - See Handbook NHB 2200.2.
- **NTIS** - Leave blank.

Block 12b. Distribution Code.

- **DOD** - Leave blank.
- **DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
- **NASA** - Leave blank.
- **NTIS** - Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

*U.S.GPO:1993-0-358-779

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

SRN In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

SRN In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

SRN In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Accession For	
NTIS GRA&I	☑
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

9/29/95

PI - Signature Date

TABLE OF CONTENTS

(5) Introduction

This grant aims at elucidating the function of the *Wnt* signaling molecules in cancer, in particular the identification of a receptor for *Wnt* proteins. *Wnt* genes encode secreted proteins involved in cell-to-cell signaling (Nusse and Varmus, 1992; McMahon, 1992). *Wnt* genes play important growth controlling roles, in particular in the mammary gland, and act as oncogenes in mouse mammary tumors (Nusse and Varmus, 1982). Little is known about the mechanism of action of *Wnt* products. Problems inherent to the nature of *Wnt* proteins have precluded the isolation and characterization of *Wnt* receptors. The isolation of these receptors is critical to our understanding of normal growth control of the mammary gland.

Using genetic and biochemical approaches generated by our work on a *Wnt* gene in *Drosophila* called *wingless* (Rijsewijk et al., 1987), we wish to identify and to clone the receptor for *wingless*. We perform genetic screens to identify suppressors and enhancers of an adult *wingless* phenotype. These modifying genes will be cloned and their properties will be examined by sequencing and transfection. We will also take advantage of our recently developed assay for soluble extracellular *wingless* protein to identify *wingless* receptors. The receptor will be characterized and using the receptor gene, we will subsequently clone mammalian *Wnt* receptors by homology.

(6) Body

6.1 Biochemistry of *wingless* signaling; characterization and isolation of the *wingless* protein.

We have developed a very useful *in vitro* assay for *wingless* signaling in our laboratory, using a cell line (clone 8 or cl-8) derived from *Drosophila* imaginal discs. These cells normally do not express *wingless* but we initially transfected cl-8 cells with constructs containing *wingless* alleles including the temperature sensitive mutant wg^{IL114}. To measure a response to *wingless*, we examined the expression of the *armadillo* protein, the most ubiquitous downstream genetic target of *wingless* during the development of the fly. We found an approximately 15-fold increase in *armadillo* protein levels at the permissive temperature (16°C) compared to non-permissive temperature (29°C) in cl-8 cells expressing wg^{IL114}.
We tested whether *wingless* from sources outside the target cells can also influence *armadillo* protein levels. In these assays, we used non-transfected cl-8 cells as targets, and *wingless* overexpressing S2 cells as donors (Cumberledge and Krasnow, 1993). These S2 cells were generated by transfection of expression constructs containing various *wingless* alleles. In the transfected S2 cells, *wingless* antigen was observed at the cell surface, in the extracellular matrix (ECM) and in a the supernatant. This protein in the medium is soluble to the extent that a 100,000 g centrifuge spin leaves the protein in the supernatant (Van Leeuwen et al., 1994).

Using co-cultured *wingless*-producing S2 cells, the ECM, and also the medium from these S2 cells, we found a large increase in *armadillo* levels in the cl-8 target cells. Serum free medium conditioned by *wingless* overexpressing S2 cells or control cells was centrifuged at 100,000g for 1 hr. and concentrated. A significant induction of *armadillo* was seen in the cl-8 cells that had been incubated with the medium. To demonstrate that the active component in the concentrated tissue culture medium from the *wingless*-producing S2 cells was indeed the *wingless* protein, we added a rabbit antiserum that was raised to a bacterial fusion protein containing

wingless protein to the medium. The antiserum removed the *armadillo*-inducing effect from the medium, whereas a control pre-immune serum had no effect.

In conclusion, we have found active soluble *wingless* protein in the medium. This protein has a rapid, concentration-dependent effect on the levels of the *armadillo* proteins. This activity can be depleted by an antibody to *wingless*, providing a quantitative and early effect of an extracellular *Wnt* protein (Van Leeuwen et al., 1994).

This assay has also been used to analyze the properties of the *wingless* protein itself. By making a series of mutant forms and analyzing the size of *wingless* protein on glycerol gradients, we found the active *wingless* protein in a higher molecular weight complex. This complex could either be a multimer of *wingless* itself or contain other, yet uncharacterized proteins. Interestingly however, *wingless* in this complex is not bound through disulfide linkages to other proteins, as non-reducing gels show a monomeric form of the *wingless* protein within this complex. Epitope tagged variants of *wingless* have been generated to purify active protein. Thus, epitopes consisting of 6 His residues (The His tag) and of the HA tag have been inserted into the *wingless* protein. We have also made variants in which a substrate site for a protein kinase has been inserted into the *wingless* protein, aiming at labeling the protein to high specific activity with radiolabelled phosphate.
These variants have been transfected into *Drosophila* Schneider S2 cells, and are like the wild type protein, secreted from cells in an active form. The *wingless* protein will be isolated by a combination of affinity, ion exchange and other chromatography methods and tested throughout these steps by the assay described before. Monoclonal antibodies against epitopes built into *wingless* will be used to generate immunoaffinity matrices. This His-tag protein can be purified by nickel-chelate chromatography. The *wingless* protein will be eluted by various methods, as mild as possible, for example by competition using the peptides used to raise the monoclonals, but otherwise by changing the pH, salt or detergent concentrations.

6.2 A genetic screen for suppressors of a *wingless* phenotype in *Drosophila*

A second route to the identification of components of *wingless* signal transduction in *Drosophila* is to take advantage of the genetic tools developed in this organism. By performing genetic screens for suppressors of a *wingless*-caused phenotype in the fly, one can uncover mutations in genes that are essential to generate this phenotype. Those genes could encode components of the *wingless* signaling pathway, including the receptor.

To create a viable, dominant adult *wingless* phenotype, we have made several P-element based constructs to obtain ectopic expression of *wingless* in larval imaginal discs, the progenitors of adult tissues. These include a construct in which *wingless* expression is driven by the *sevenless* promoter, pSEW-*wingless*, which is known to be active only in the eye imaginal disc. The transgenic flies that were obtained have a very specific phenotype in the eye: an almost complete absence of interommatidial bristles. Normally these bristles arise throughout most of the eye in a regular pattern. While there are approximately 600 bristles in the wild-type eye, the pSEW-*wingless* flies have 0-10 bristles. Other structures in the eye and elsewhere are apparently normal. This phenotype is 100% penetrant and easy to score with a dissecting microscope.

Transgenic flies carrying a sevenless-driven temperature sensitive allele of *wingless* (pSEW-*wingless*(ts)) display the loss-of-bristle phenotype in a temperature-dependent manner. When this temperature dependent transgene is combined with a loss of function mutation in *armadillo* , the phenotype becomes suppressed. At 17.9°C approximately 200-300 bristles per eye form. When the dose of *armadillo* gene is reduced by a factor of two (flies heterozygous for a *armadillo* mutation), the number of bristles significantly increases. Thus, a reduction of *armadillo* gene activity can suppress the pSEW-*wingless*(ts) phenotype, suggesting that other components of the *wingless* -signaling pathway can be identified with this assay (Noordermeer et al., 1994). Moreover, this phenotype is also generated by a *wingless* temperature sensitive allele, but in a temperature dependent manner. The phenotype is fully

penetrant, as no wild type eyes have been detected at permissive or intermediate temperatures.

This penetrant adult viable *wingless* phenotype has been used to perform a genome wide screen for dominant suppressors or enhancers of *wingless*. The principle behind this screen is to search for mutations that will give a phenotype when one allele has undergone a loss-of-function mutation. Normally, complete absence of one allele will not give a phenotype. But in a genetic background where the phenotype of one gene (in this case *wingless*) is dosage-sensitive, absence of one copy of an interacting gene may modify this phenotype. These screens is been done in the F1 generation, so provided the phenotype to be used is easily scored under a dissection microscope (as the pSEW-*wingless*(ts) bristle phenotype is), large (10^5) numbers of mutagenized chromosomes can be screened. Especially since the phenotype is semi-quantitative (i.e. the number of bristles on the eye can be approximated) this screen is very sensitive to dosage of gene products interacting with *wingless*.

An advantage of the screen is that not only suppressors but also enhancers of the pSEW-*wingless*(ts) phenotype can be scored. By performing the crosses at an intermediate temperature, where about 30% of the normal number of bristles are formed, modifications resulting in either fewer (enhancers) or more (suppressors) bristles were identified. Thus, if the *wingless* receptor acts negatively in the absence of ligand, and this negative effect is relieved by binding of *wingless*, the genetic screen may still uncover a mutation in its gene.

We have now isolated approximately 20 suppressors and enhancers of the *wingless* phenotype in the eye. These genes have been mapped and have been assembled into complementation groups, a prerequisite for molecular cloning. We have also performed clonal analysis of these genes, indicating that some of them also have a phenotype in the homozygous state. The mapping data will now be used to start cloning these modifying genes.

(7) Conclusions

Since the work started, approximately one year ago, we have made significant progress on both lines in the original grant application. We have started the purification of the *wingless* protein, and we have isolated a fairly large number of genes modifying *wingless* signaling in *Drosophila*.

(8) References

Cumberledge, S. and Krasnow, M. A. (1993). Intercellular signalling in *Drosophila* segment formation reconstructed *in vitro*. Nature *363*, 549-552.

McMahon, A. P. (1992). The *Wnt* family of developmental regulators. Trends Genet. *8*, 236-242.

Noordermeer, J., Klingensmith, J., Perrimon, N. and Nusse, R. (1994). *dishevelled* and *armadillo* act in the *wingless* signalling pathway in *Drosophila*. Nature *367*, 80-83.

Nusse, R. and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell *31*, 99-109.

Nusse, R. and Varmus, H. E. (1992). Wnt Genes. Cell *69*, 1073-1087.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987). The *Drosophila* homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene *wingless*. Cell *50*, 649-657.

Van Leeuwen, F., Harryman Samos, C. and Nusse, R. (1994). Biological activity of soluble *wingless* protein in cultured *Drosophila* imaginal disc cells. Nature *368*, 342-344.